

Lessons From the First Comprehensive Molecular Characterization of Cell Cycle Control in Rodent Insulinoma Cell Lines

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OBJECTIVE—Rodent insulinoma cell lines may serve as a model for designing continuously replicating human β -cell lines and provide clues as to the central cell cycle regulatory molecules in the β -cell.

RESEARCH DESIGN AND METHODS—We performed a comprehensive G1/S proteome analysis on the four most widely studied rodent insulinoma cell lines and defined their flow cytometric profiles and growth characteristics.

RESULTS—1) Despite their common T-antigen–derived origins, MIN6 and BTC3 cells display markedly different G1/S expression profiles; 2) despite their common radiation origins, RINm5F and INS1 cells display striking differences in cell cycle protein profiles; 3) phosphorylation of pRb is absent in INS1 and RINm5F cells; 4) cyclin D2 is absent in RINm5F and BTC3 cells and therefore apparently dispensable for their proliferation; 5) every cell cycle inhibitor is upregulated, presumably in a futile attempt to halt proliferation; 6) among the G1/S proteome members, seven are pro-proliferation molecules: cyclin-dependent kinase-1, -2, -4, and -6 and cyclins A, E, and D3; and 7) overexpression of the combination of these seven converts arrested proliferation rates in primary rat β -cells to those in insulinoma cells. Unfortunately, this therapeutic overexpression appears to mildly attenuate β -cell differentiation and function.

CONCLUSIONS—These studies underscore the importance of characterizing the cell cycle at the protein level in rodent insulinoma cell lines. They also emphasize the hazards of interpreting data from rodent insulinoma cell lines as modeling normal cell cycle progression. Most importantly, they provide seven candidate targets for inducing proliferation in human β -cells. *Diabetes* 57:3056–3068, 2008

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Nutrients, growth factors, and physiological states such as obesity and pregnancy can induce β -cell replication. However, the molecular pathways that regulate DNA synthesis and mitosis in β -cells have only recently begun to be elucidated. Most insight into pancreatic β -cell cycle control is derived from studies in mice with targeted mutations in either the inhibitors or activators of cell cycle progression. For example, inactivating mutations in several cell cycle control molecules have revealed roles for p27, p18, p16, menin, pRb, p53, cyclin D1, cyclin D2, cdk4, E2F1, and E2F2 (1,2). These studies suggest that the G1/S checkpoint is especially important for the control of the cell cycle in pancreatic β -cells.

Other attractive models in which to study β -cell cycle replication are rodent insulinoma cell lines, which are widely used as models for β -cell signaling, function, and replication (3). The four cell lines most widely used in β -cell research are MIN6, BTC3, INS1, and RINm5F cells. MIN6 and BTC3 cells are related lines that were generated by transgenic overexpression of the large T-antigen of the SV40 virus in mouse β -cells (3,4,5). INS1 and RINm5F are also related cell lines that were derived from radiation-induced tumors in rats (3,6,7). These cell lines vary in characteristics, but all are at least partially differentiated and proliferate more rapidly than normal β -cells. Surprisingly, despite their extensive use in the field of β -cell biology, there is almost no information regarding the mechanisms that lead to their loss of normal cell cycle control (3–7).

The lack of a continuously growing human β -cell line suggests the possibility that lessons learned from rodent insulinoma lines may offer clues on how to generate corresponding human β -cell lines that may be useful for research and for cell replacement therapy of diabetes. We therefore examined the G1/S proteome of these four rodent insulinoma cell lines and compared them with primary islets in hope that these cell lines might reveal common mechanisms that lead to continuous proliferation of insulin-secreting cells and potential targets to induce proliferation in normal β -cells.

As has been described previously in most human cancers (8–10), comprehensive analysis revealed multiple abnormalities in G1/S control in each of the four cell lines. Interestingly, despite their common origins (X-radiation in the rat cell lines or T-antigen overexpression in the mouse lines), insulinomas of common origin display very different G1/S profiles. In addition, several

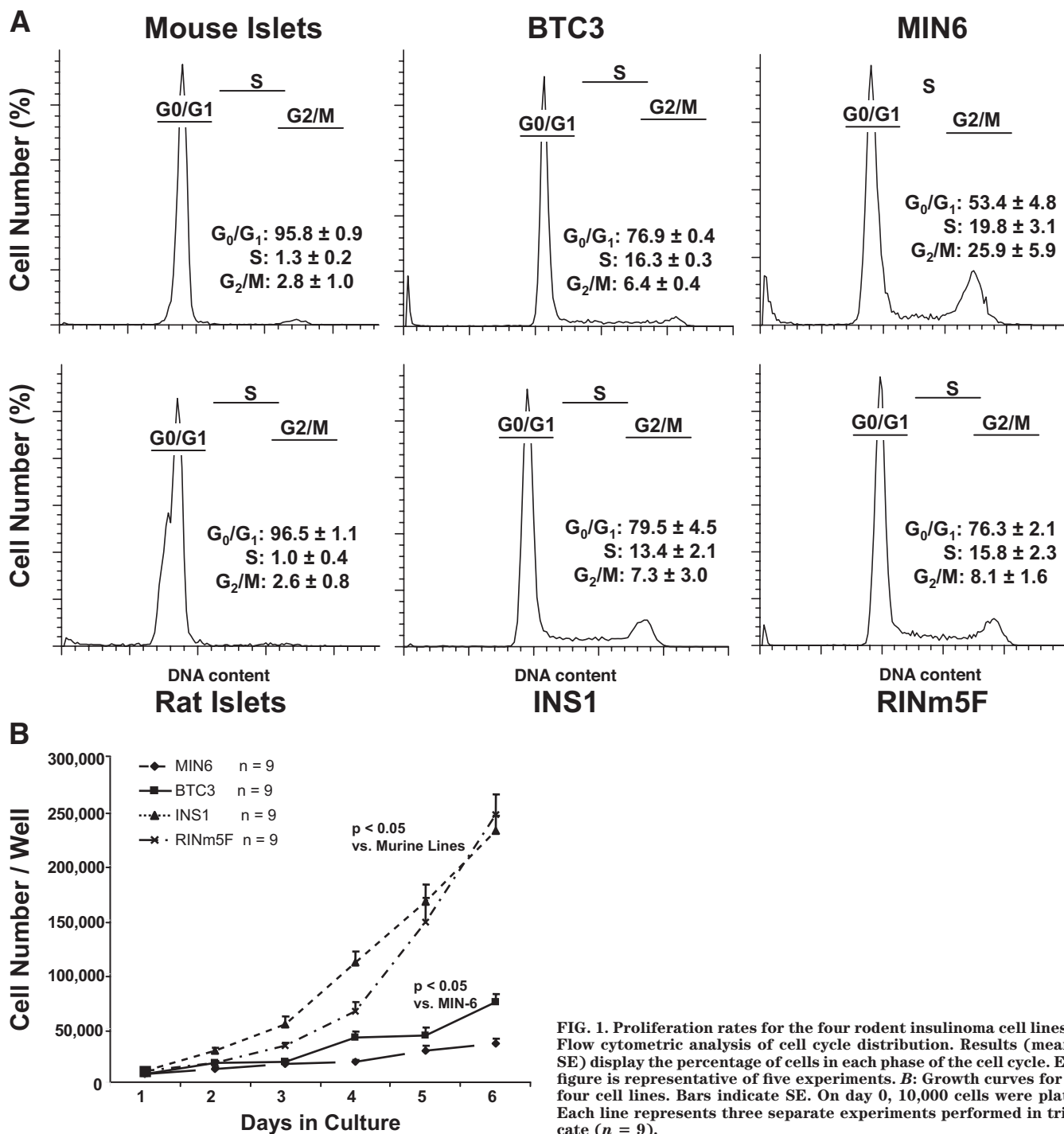


FIG. 1. Proliferation rates for the four rodent insulinoma cell lines. **A:** Flow cytometric analysis of cell cycle distribution. Results (mean \pm SE) display the percentage of cells in each phase of the cell cycle. Each figure is representative of five experiments. **B:** Growth curves for the four cell lines. Bars indicate SE. On day 0, 10,000 cells were plated. Each line represents three separate experiments performed in triplicate ($n = 9$).

molecules that appear to be critical in maintaining arrest or allowing cell cycle progression in normal rodent islets, e.g., cyclin D2, are entirely dispensable in mouse and rat insulinoma lines. Finally, seven G1/S molecules that are capable of stimulating β -cell cycle progression were identified. These observations have important cautionary implications for the use of the four cell lines as models of "normal" β -cell replication. They also suggest strategies for developing continuously growing human β -cell lines.

RESEARCH DESIGN AND METHODS

Cell lines. BTC3, MIN6, INS1, and RINm5F cells were obtained from Dr. Lydia Aguilar-Bryan (PNRI, Seattle, WA), Susumu Seino (Kobe University,

Kobe, Japan), Dr. Doris Stoffer (University of Pennsylvania, Philadelphia, PA), and American *Type Culture* Collection, respectively. They were uniformly studied at 80% confluence. Each cell line was cultured in its standard tissue culture medium: BTC3 and MIN6 in Dulbecco's modified Eagle's medium (20 mmol/l glucose with 10% FCS); and INS1 and RINm5F in RPMI (11 and 5.5 mmol/l glucose, respectively, and 10% FCS; Invitrogen, Carlsbad, CA). MIN6 cells require 50 μ mol/l β -mercaptoethanol, and INS1 cells require 10 mmol/l HEPES, 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, and 50 μ mol/l β -mercaptoethanol.

Cell cycle distribution and cell growth. Trypsinized cells from cell lines and isolated islets were fixed with 70% ethanol and analyzed using flow cytometry (fluorescence-activated cell sorting [FACS]). On the day of FACS, fixed cells were pelleted and resuspended in a PBS solution containing 50 μ g/ml propidium iodide (Sigma, St. Louis, MO), 100 units/ml RNase A (Sigma), and 1 g/l glucose. Stained cells were filtered through a 30- μ m nylon mesh, and DNA content was analyzed on a flow cytometer (Becton Dickinson, Franklin

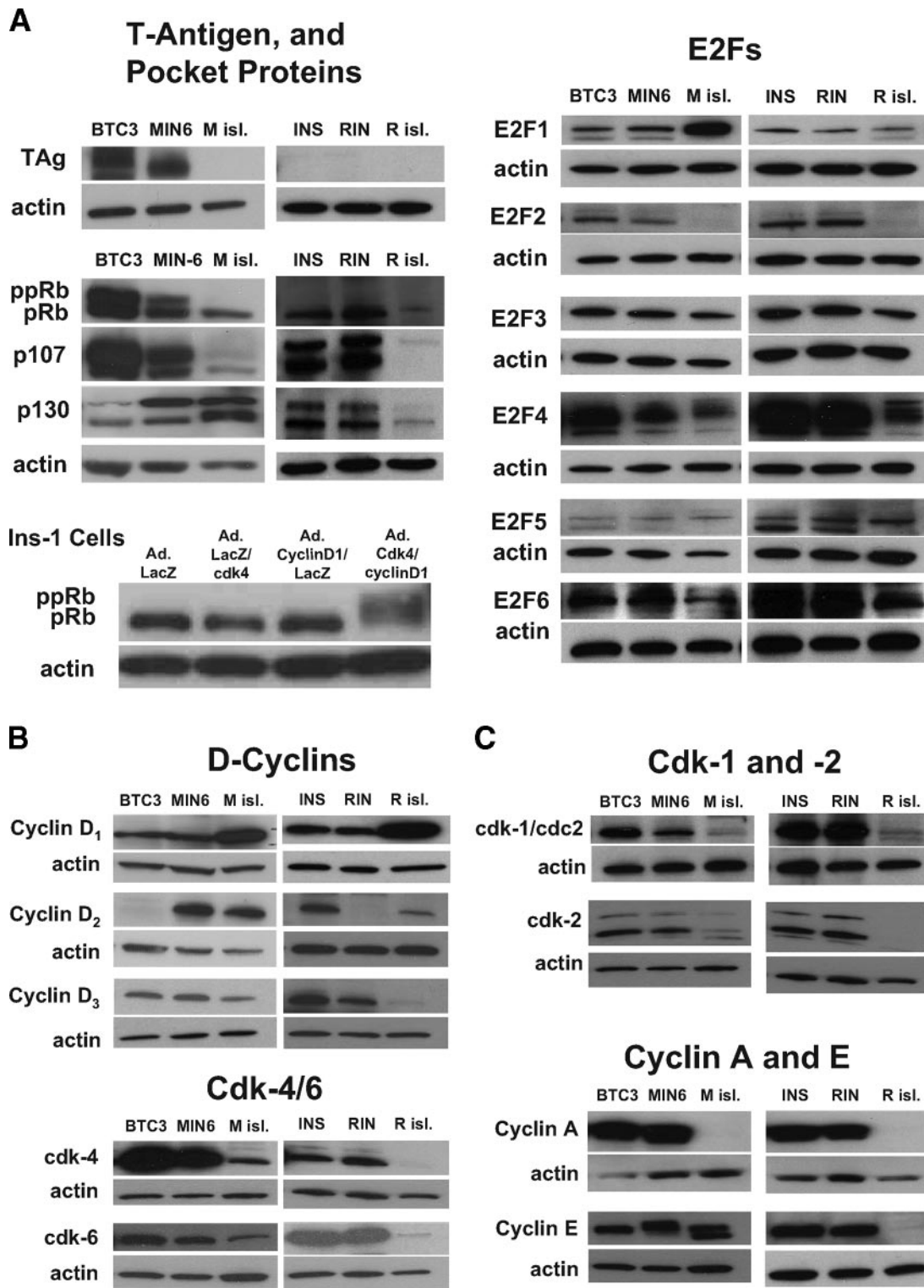


FIG. 2. The G1/S proteome of the four rodent insulinoma cell lines. MIN6 and BTC3 cell lines are compared with mouse islets (M isl). INS1 (INS) and RIN (RINm5F) lines are compared with rat islets (R isl). Actin blots are representative and demonstrate even loading. **A:** Western-blot analysis of T-Ag, pocket proteins, and the E2F transcription factor family. The *bottom left panel* shows induction of pRb phosphorylation in INS1 cells that have been transduced with the combination of the adenoviruses Ad.cdk4 plus Ad.cyclin D1. **B:** The cyclin D1, D2, and D3 and cdk-4 and -6 family. **C:** The cdk-1/cdc2 and cdk-2 and cyclin A and E family. **D:** The INK4 family, the CIP/KIP family, and the tumor suppressor, menin. **E:** The tumor suppressor, p53, its regulatory protein MDM2, and the Id family. Each blot represents three to five immunoblots.

Lakes, NJ). To examine growth, 10,000 cells were plated per well in 24-well tissue culture plates. Cells were counted using a hemocytometer in triplicate every day for 6 days after initial plating.

Adenoviral production. Adenoviruses (Ad.5 or pAd-Easy system) were prepared according to methods that we have previously described in detail (11–14). Multiplicity of infection (MOI) was determined by optical density at 260 nm and by plaque assay.

Islet isolation, adenoviral transduction, and glucose-stimulated insulin secretion. All animal studies were performed in compliance with and with approval of the University of Pittsburgh Institutional Care and Use Committee. Islets were isolated from 8- to 10-week-old Sprague-Dawley rats (Taconic, Germantown, NY) and 8- to 12-week-old CD1 and C57Bl6 mice (The Jackson Laboratories, Bar Harbor, ME) as previously described (14,15). Isolated rat islets were cultured in RPMI medium supplemented with 10% FCS, 5.5 mmol/l

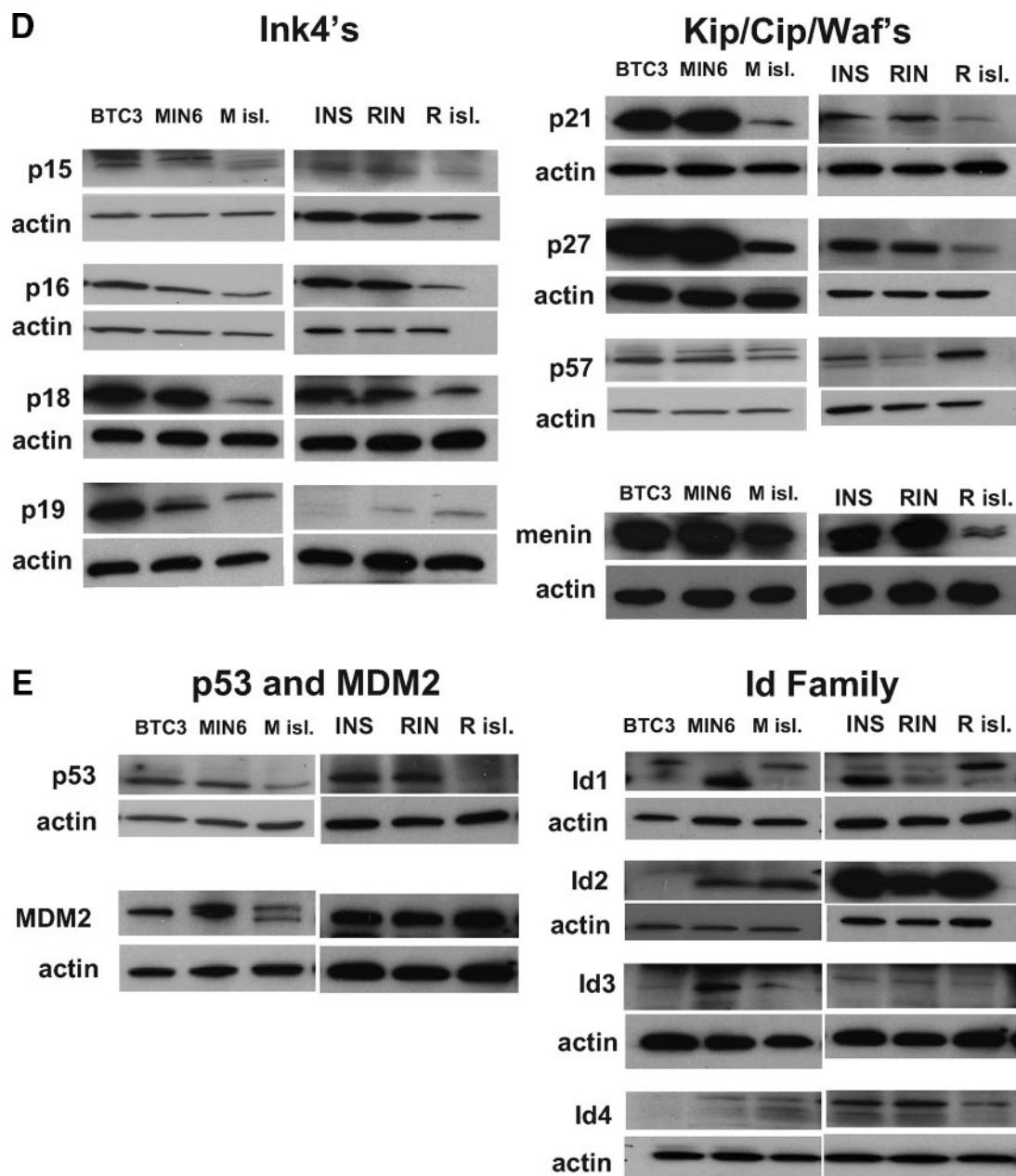


FIG. 2. Continued

glucose, and penicillin-streptomycin for 24 h before adenoviral transduction. Islets were transduced in serum-free medium for 1 h at 37°C with adenoviruses containing the cDNAs for green fluorescent protein (GFP); human cdk-1, -2, -4, and -6; or cyclin A, D1, and E individually or in combination ("Combo") as detailed in RESULTS and in the figure legends. Islets were then incubated for 72 h in RPMI supplemented with 10% FCS and 5.5 mmol/l glucose, unless otherwise stated. For glucose-stimulated insulin secretion (GSIS), static assays were performed as described previously (11,14,16,17) using the ALPCO (Salem, NH) ultrasensitive insulin enzyme immunoassay. Matched islet equivalents (IE) were hand-picked on the day of GSIS, 48 h after adenoviral transduction.

Western blot analysis. Protein extracts and immunoblotting of the four cell lines as well from mouse and rat islets (24 h after isolation) were performed as reported previously (16,17) using previously published primary and secondary antibodies. Fresh membranes were used for immunoblots for all G1/S members.

Quantification of β -cell proliferation and cell death. BrdU (10 μ g/ml; Sigma) was added to the culture media 24 h before fixation. Islets were fixed in Bouin's solution for 1 h, washed with water, and then stored in 10% neutral buffered formalin until embedding. To quantitate β -cell proliferation, 5- μ m sections were double-stained with anti-insulin (Dako, Carpinteria, CA) and anti-Ki-67 (Lab Vision, Fremont, CA) or anti-BrdU (Abcam, Cambridge, MA).

To determine β -cell death, islet sections were stained for apoptotic cells using terminal deoxynucleotidyl-mediated dUTP nick-end labeling (TUNEL) (Roche, Indianapolis, IN) and anti-insulin.

Real-time PCR. Quantitative PCR was performed using standard methods and an ABI 7300 Real-Time PCR System. The relative quantification for a given gene was corrected to the β -actin mRNA values. The sequences of the primers are included in supplemental Table 1, which is available in an online appendix at <http://dx.doi.org/10.2337/db08-0393>.

Islet transplant studies. Rat islets were transduced 24 h after the isolation; 24 h after transduction, the islets were transplanted into NOD-SCID mice using procedures previously described (11). One rat IE was defined as being 125 μ m in diameter.

Statistics. For all studies, Student's two-tailed unpaired *t* test was performed using Microsoft Excel. *P* values <0.05 were considered to be significant. Bars in the figures indicate SE.

RESULTS

Growth characteristics of BTC3, MIN6, INS1, and RINm5F cells. To confirm that the four insulinoma cell lines replicate more rapidly than normal rodent β -cells, the three cell cycle phases (G0/G1, S, and G2/M) were exam-

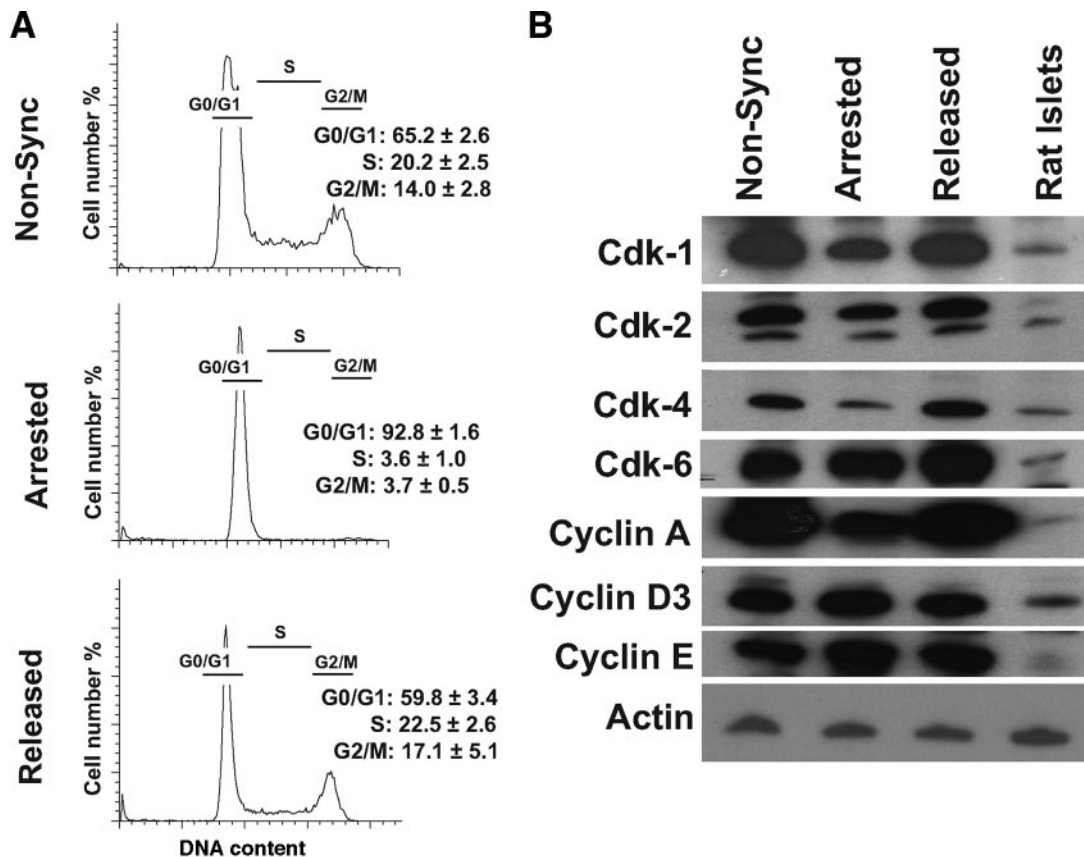


FIG. 3. The effect of cell cycle phase on the seven key pro-cell cycle molecules in INS1 cells. **A:** FACS histogram of INS1 cells grown under randomly cycling nonsynchronized (Non-Sync) conditions, under conditions of growth arrest (Arrested) induced by exposure to 2.8 mmol/l glucose and serum withdrawal for 96 h, and after release from arrest (Released) by the addition of complete medium (see RESEARCH DESIGN AND METHODS) for 24 h. **B:** Representative immunoblots of the seven key G1/S molecules under each of these three conditions, compared with extracts of rat islets. As can be seen, whereas some changes do occur in the G1/S molecules with growth arrest, they are minor compared with their marked overexpression compared with rat islets.

ined by FACS. The two murine insulinoma lines (BTC3 and MIN6) were compared with isolated primary mouse islets, whereas the two rat lines (RINm5F and INS1) were compared with isolated primary rat islet cells. Unlike primary islet cells, which are arrested in G0/G1 (96%) (1,2,18), the insulinoma cell lines showed a decreased G0/G1 population and an increased proportion (24–46%) of cells actively engaged in the cell cycle (S and G2/M) (Fig. 1A).

This enhanced cell cycle progression was confirmed by analysis of the growth curves of the four cell lines (Fig. 1B). By day 6, the two rat cell lines were expanded 24- to 25-fold compared with an only 4- to 6-fold increase for the mouse cell lines.

Comprehensive analysis of the G1/S proteome in the four insulinoma cell lines. As detailed in Fig. 2A, large T-antigen was expressed in the two mouse lines, BTC3 and MIN6 cells, consonant with the use of T-antigen to derive these cells (4,5). As expected, the normal rat and mouse islets and the two rat insulinoma lines lacked T-antigen. The pocket proteins, pRb, p107, and p130 that serve as the final common pathway to G1/S control (Fig. 2A, middle left panel), all were present in the four insulinoma cell lines. In contrast to an anticipated increase in pRb phosphorylation in all four lines, phospho-pRb was present in BTC3 cells and absent in INS1 and RINm5F cells. Moreover, pRb and p107 appeared to be expressed at higher levels in the insulinoma cell lines than normal islets. Higher-molecular weight bands present in the p107 and p130 blots may represent phosphorylated forms. As

can be seen in Fig. 2A (bottom left panel), pRb in INS1 cells was readily phosphorylated by overexpression of cdk-4 and cyclin D1, indicating that there are no intrinsic abnormalities in pRb that prevent its phosphorylation. Thus, the pathophysiology underlying the changes in the pocket proteins remains unexplained for any of the four insulinoma cell lines.

Figure 2A, right panel, displays for the first time the E2F family in the four cell lines. All six appear to be present in the cell lines. Several, such as E2F2, -4, and -6, appear to be expressed at higher levels in the cell lines than in primary islets. Conversely, E2F1 appeared to be reduced in the murine cell lines compared with normal islets.

The D-cyclins and their cognate cyclin-dependent kinases (cdks) are shown in Fig. 2B. Cyclin D1, reported to be increased in human insulinoma (19), was reduced in all four rodent cell lines. Cyclin D2, essential for the murine β -cell cycle progression (20,21), was undetectable in two of the four cell lines, BTC3 and RINm5F cells. Interestingly, despite their common T-antigen origins, the two murine cell lines displayed opposite cyclin D2 profiles: BTC3 lacked cyclin D2, whereas MIN6 cells expressed easily detectable cyclin D2. Similarly, despite their common radiation-induced pathogenesis, the same was observed for INS1 versus RINm5F cells. Finally, cyclin D3, generally felt to be the least important of the three D cyclins (21,22), was substantially increased in the two rat cell lines.

Figure 2B also demonstrates that cdk-4 and cdk-6 are unequivocally increased in all four insulinoma cell lines.

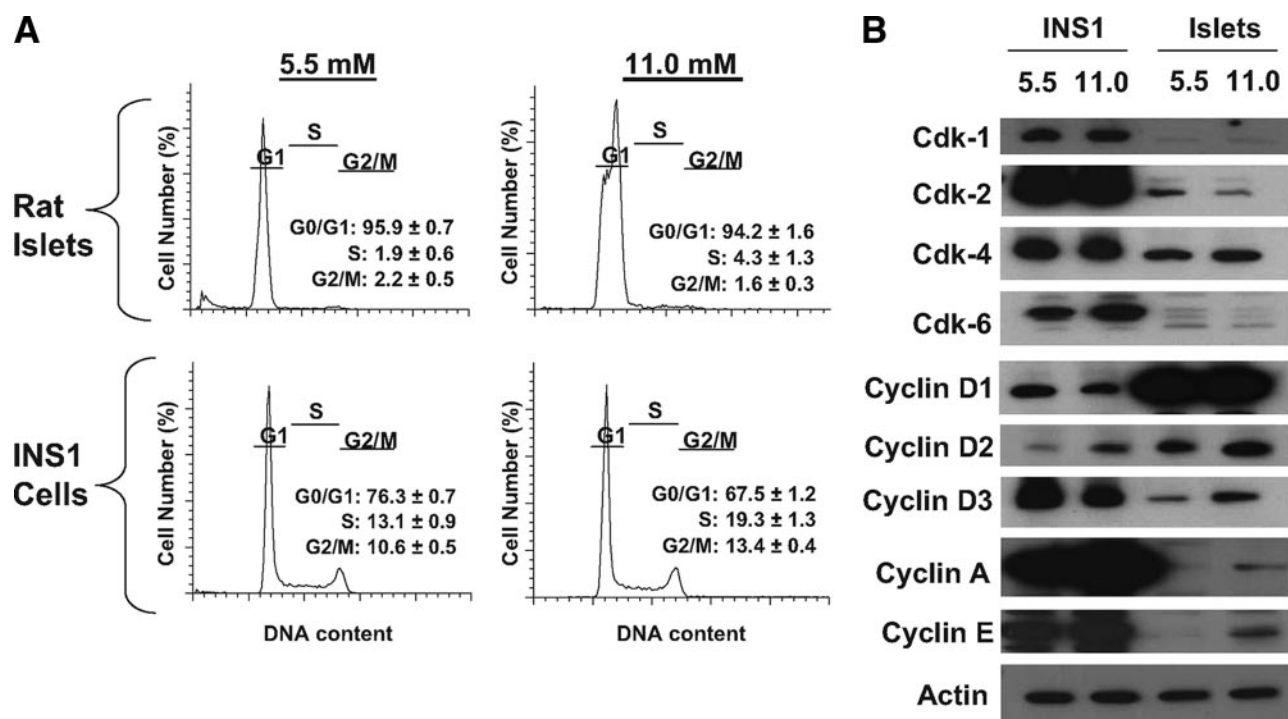


FIG. 4. The effect of glucose on key cell cycle proteins in rat islets and INS1 cells. *A*: Cell cycle distribution of rat islet cells and INS1 cells at 5.5 and 11 mmol/l glucose. *B*: The effect of glucose on the expression of the seven candidate cell cycle activators plus cyclins D1 and D2. 5.5 and 11 refer to 5.5 and 11 mmol/l glucose concentrations. Each experiment was performed three times.

Figure 2C shows that the same is true for cdk-1 and cdk-2 and for their cognate partners, cyclin E and A. Thus, of the seven potential G1/S cyclins and cdks (cdk1, -2, -4, and -6 and cyclins A, D, and E) all seven are increased in each of the four cell lines.

The INK4 and KIP/CIP/WAF families are inhibitors of the cyclin-cdk proteins. Figure 2D demonstrates that many of these are increased in the four insulinoma cell lines. In addition, the β -cell cycle inhibitor, menin, which transcriptionally activates p18Ink4 and p27kip, is present and is increased in the two rat cell lines. Similarly, the tumor suppressor p53 is increased in all four cell lines. Its E3 ligase, MDM2, was increased in the mouse lines but unaltered in the rat lines (Fig. 2E). Finally, the four Id proteins, which interact with the pocket proteins to regulate their DNA binding and differentiation (23), were variably present but showed no consistent pattern.

The increase in the seven key G1/S molecules is independent of cell cycle phase. The seven "candidate cell cycle activators" in Fig. 2B and C served as the focus of the remainder of the study. To determine whether the increase in the seven key G1/S molecules was an intrinsic feature on the insulinomas or reflected only certain cell cycle phases in randomly cycling cells, we compared their level of expression in randomly cycling, starvation-arrested, and resynchronized INS1 cells to levels observed in isolated rat islets. As can be seen in Fig. 3, although there were minor changes among the former three conditions, under all conditions, levels were markedly higher in INS1 cells than in rat islets.

Effect of different glucose concentrations on the expression of the seven candidate cell cycle activators. Because each of the cell lines was grown using different glucose concentrations and because glucose is known to drive rodent β -cell replication (24–29), determining whether any of the cdk or cyclin changes observed above reflected culture conditions rather than intrinsic

insulinoma biology was important. We therefore compared the cell cycle distribution of the INS1 cells and primary rat islets cultured in 5.5 versus 11 mmol/l glucose (Fig. 4A). In normal rat islet cells, S phase was approximately doubled in 11 compared with 5.5 mmol/l glucose, although this difference did not achieve statistical significance. Thus, as anticipated, higher glucose levels favor β -cell replication. The expression of cyclins and cdks were studied in INS1 cells and rat islet cells in both glucose concentrations. Cyclins A, E, and D3 were found to be upregulated in normal islet cells by higher glucose concentration, but the expression did not approach the levels of expression of these proteins observed in INS1 cells (Fig. 4B). Thus, although glucose can stimulate proliferation, it is not the cause of the striking upregulation of the seven candidate cell cycle activators. Rather, these are intrinsic features of the four cell lines.

Overexpression of the seven candidate cell cycle activators mimics proliferation rates in INS1 cells. To determine whether any of the seven was capable of inducing cell cycle progression, they were overexpressed individually in isolated primary rat islets using adenovirus. "Ad.cyclin D" in the experiments refers to cyclin D1. Figure 5A confirms that each of the seven proteins was overexpressed by the appropriate adenovirus. The effect of overexpression of each of the seven molecules on islet cell proliferation was studied by FACS. Modest but statistically significant increases in proliferation were observed when cyclin E or cyclin D was overexpressed individually but not by the overexpression of the other proteins (Fig. 5B). In contrast, the maximum rate of proliferation achieved using combinations of cell cycle agonists closely resembled that seen in INS1 cells (Fig. 5C). Therefore, the seven cell cycle combination (Ad.Combo) was used for subsequent experiments. In a dose-response study, the minimal MOI of the Ad.Combo that was able to induce maximal proliferation was 250 or lower (Fig. 5D).

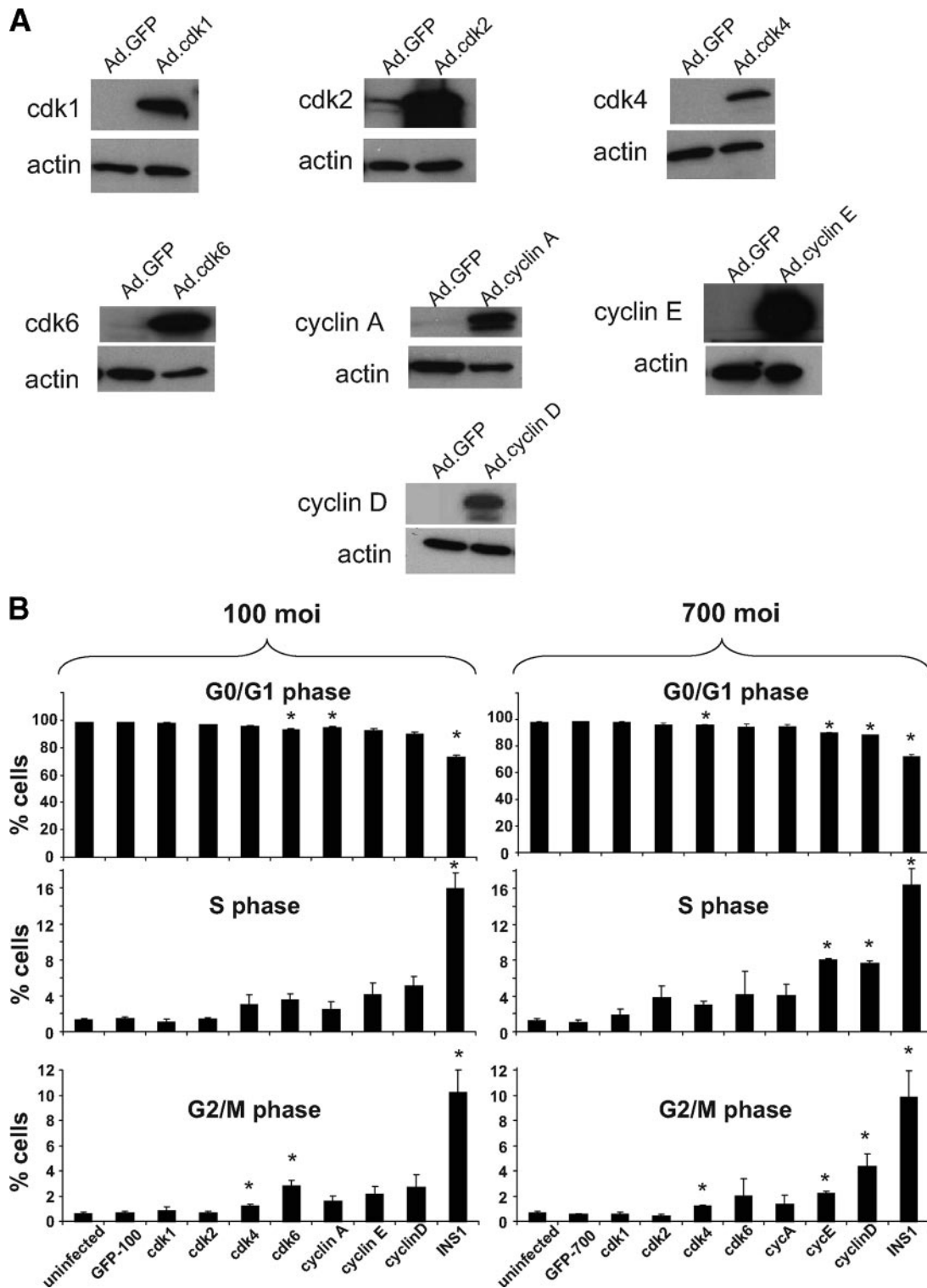


FIG. 5. Consequences of cyclin-cdk overexpression in rat islets. **A:** Documentation of overexpression of the seven candidate cell cycle activators. **B:** The effect of individual overexpression of the seven cell cycle activators at 100 MOI (left) or 700 MOI (right) on rat islet cell proliferation as measured by flow cytometry. * $P < 0.05$. Each experiment was performed three times. **C:** Flow cytometric analysis of the effect of combined overexpression of the seven cell cycle activators on rat islet cell proliferation. Each condition used a maximum of 700 MOI, with 100 MOI of each virus shown on the x-axis. * $P < 0.05$ vs. Ad.GFP; # $P < 0.05$ compared with INS1 cells. Each experiment was performed three to five times. **D:** The effect of three different adenovirus MOI on rat islet cell proliferation. The numbers of MOI used for these experiments were 250, 500, and 700 MOI. Each bar represents four experiments.

Insulin and Ki-67 double immunofluorescent staining confirmed that proliferation was occurring in β -cells: An average of $26 \pm 3.6\%$ of β -cells were proliferating, a 13-fold increase compared with Ad.GFP or nontransduced controls (Fig. 6A). In parallel, BrdU staining revealed comparable results: An average of $20 \pm 4.1\%$ of β -cells were BrdU

positive, a sevenfold increase compared with Ad.GFP control (Fig. 6B).

Cell cycle-driven β -cell proliferation does not induce cell death. To establish whether overexpression of the seven candidate activators induced apoptosis, we double-stained sections of rat islets transduced with Ad.GFP, Ad.Combo, or

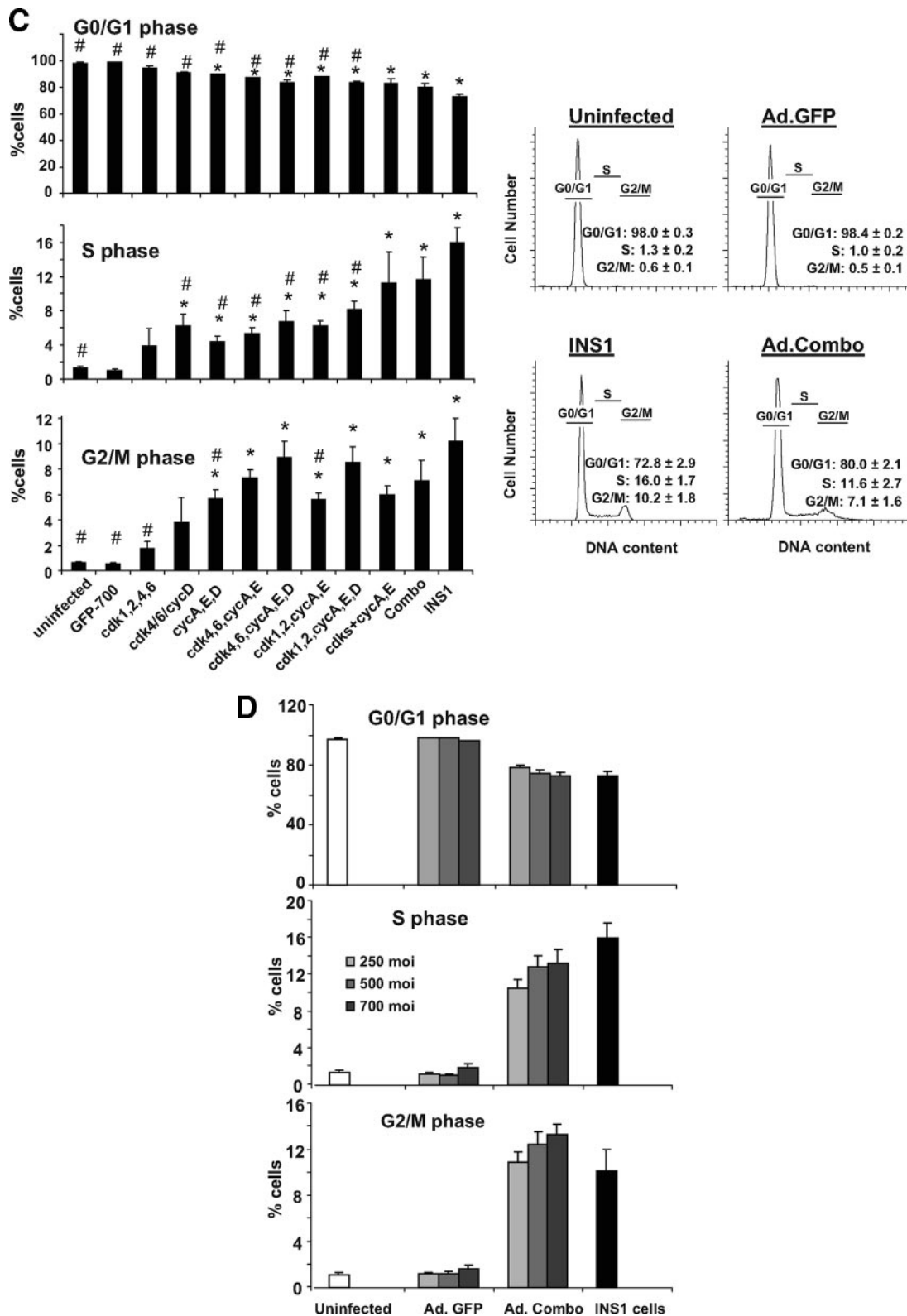


FIG. 5. Continued

nontransduced controls for TUNEL and insulin. The number of cells doubly positive for insulin and TUNEL was low: 1.03 ± 0.9 , 1.34 ± 0.7 , and $0.85 \pm 0.5\%$ in control, Ad.GFP, and Ad.Combo transduced islets, respectively (Fig. 7).

Accelerated β -cell proliferation may be associated with mild dedifferentiation. To investigate whether cell cycle activators led to dedifferentiation in rat β -cells

(23,30), we examined β -cell differentiation markers in rapidly proliferating Ad.Combo-transduced primary rat islets and compared them with Ad.GFP-transduced and nontransduced control islets. As shown in Fig. 8A, of the five β -cell-specific mRNAs examined (insulin, Glut2, Pdx1, Sur1, and Kir6.2) and glucagon, no significant differences were observed between Ad.Combo-transduced islets ver-

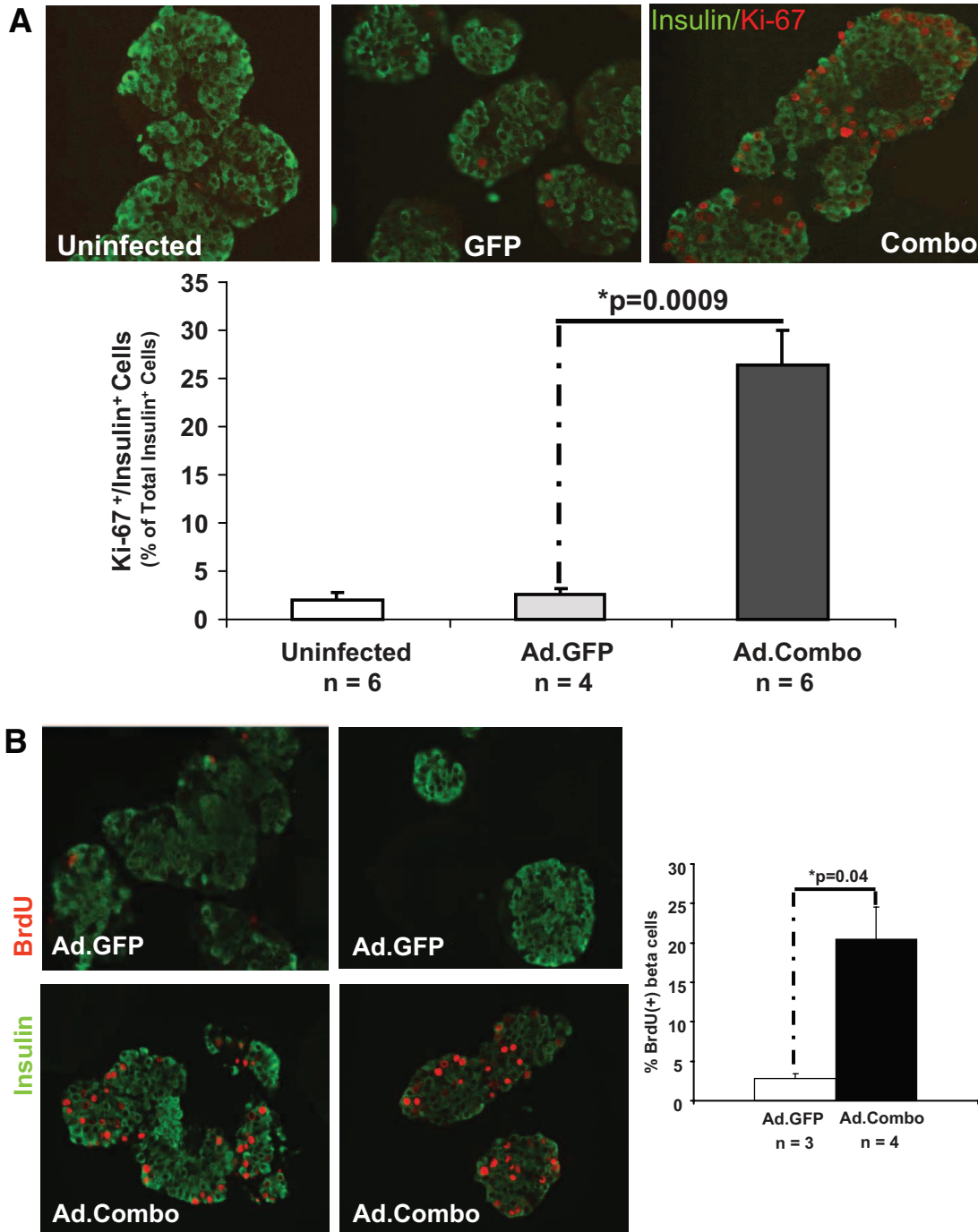


FIG. 6. The effect of combined overexpression of the seven cell cycle activators on rat β -cell proliferation. β -Cell proliferation was measured by double staining for Ki-67 and insulin (A) or BrdU and insulin (B). Representative examples are shown in the top panels and quantitation in the bottom panel and represented by percentage of double positives Ki-67/insulin or BrdU/insulin. *n* indicates the number of experiments performed. (Please see <http://dx.doi.org/10.2337/db08-0393> for a high-quality digital representation of this figure.)

sus Ad.GFP controls. However, adenoviral transduction itself did reduce Pdx1, Kir6.2, and glucagon in Ad-transduced islets.

GSIS studies (Fig. 8B) demonstrated robust insulin secretion from uninfected and Ad.GFP-transduced islets. Ad.combo-transduced islets displayed a trend toward higher basal insulin secretion and lower GSIS, but these differences from the two control groups were not significant.

Ad.combo-transduced islets function normally in vivo. The studies reported this far suggested that Ad.combo-transduced islets might be slightly dedifferentiated (Fig. 8A and B) or, alternatively, because of their accelerated

proliferation, might have an engraftment or functional advantage in vivo. To explore these possibilities more definitively, transplant of Ad-transduced rat islets into streptozotocin-induced diabetic NOD-SCD mice was performed (Fig. 8C). Sham-transplanted animals displayed glucoses in the 400–500 mg/dl range for the 4 weeks of the study. Animals receiving 200 control IE displayed near-normal postprandial glucose values, whereas those receiving 100 and 50 control IE displayed progressively poorer glycemic control. Importantly, animals receiving equivalent numbers (50, 100, or 200) of rat IE transduced with the Ad.combo performed neither better nor worse than con-

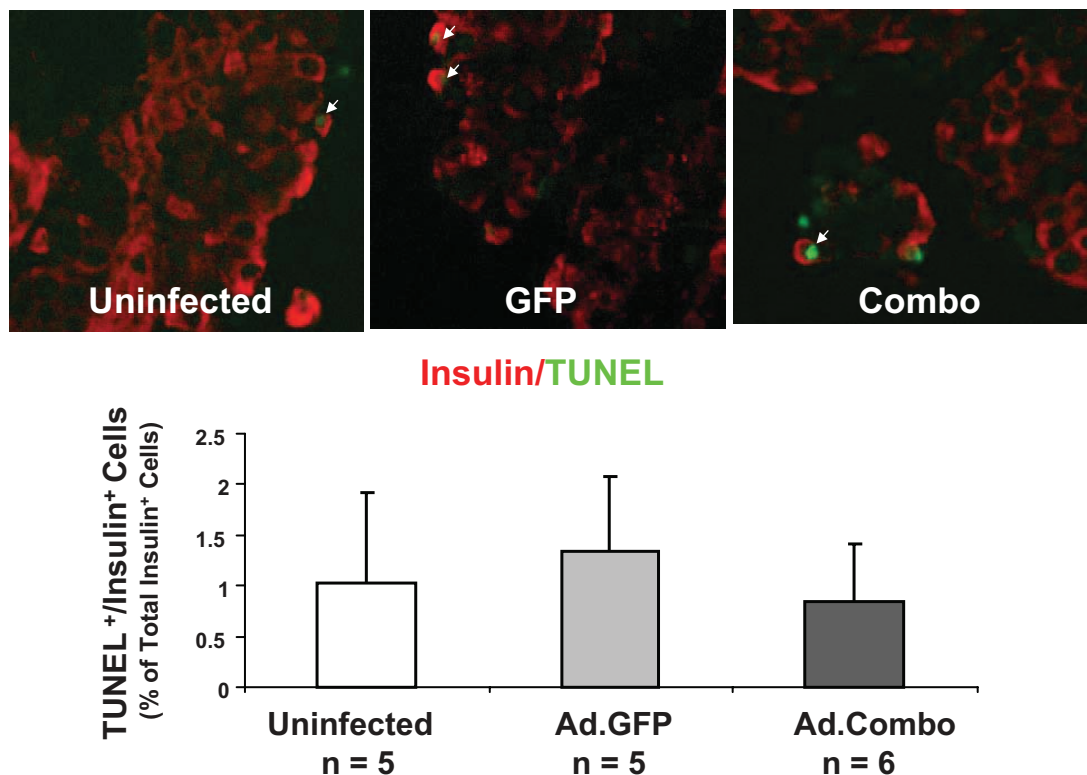


FIG. 7. The effect of combined overexpression of the seven cell cycle activators on rat β -cell death. β -Cell apoptosis was measured by double staining of TUNEL and insulin and represented by percentage of double positives for TUNEL/insulin. The conditions were the same as in Fig. 4C, and fixation was performed 72 h after transduction. *n* indicates number of experiments performed. (Please see <http://dx.doi.org/10.2337/db08-0393> for a high-quality digital representation of this figure.)

tol islets. These results can be interpreted to indicate that although Ad.combo did not enhance functional engraftment, there was no net increase or decrease in β -cell differentiation. However, without defining β -cell mass in the islet grafts, it is possible that Ad.combo-induced proliferation in vivo expanded β -cell mass substantially, masking a reduction in β -cell differentiation or function.

DISCUSSION

This study represents the first comprehensive molecular characterization of G1/S cell cycle regulation in continuously proliferating β -cell lines. We have 1) identified striking differences in cell cycle control molecules in insulinoma cell lines compared with primary β -cells; 2) identified seven candidate cell cycle activators that may contribute to cell cycle progression in rodent insulinoma cell lines; 3) formulated a strategy to dramatically stimulate proliferation of primary rat β -cells to the level observed in insulinoma cell lines; 4) shown that this does not enhance cell death or greatly impair differentiation or function in vitro; and 5) shown that transduction with seven cell cycle activators neither enhances nor attenuates rat islet function in vivo. These observations may be directly applicable to efforts to create continuously growing human β -cell lines and to expand human β -cell mass for β -cell replacement treatment of diabetes.

Despite the fact that rodent insulinoma cell lines were developed 20–30 years ago (3–7), the molecular events responsible for their ability to replicate continuously for decades remain entirely unknown. We have observed that the large majority of rat and mouse islet cells are in G0/G1, which agrees nicely with data published using other

techniques, confirming the slow rate of normal β -cell proliferation (18). In contrast, for rodent insulinoma cells, a substantial proportion are not arrested but are instead actively cycling. The rat lines, INS1 and RINm5F, replicate far more rapidly than the mouse lines, MIN6 and BTC3. Whether this difference in proliferation rate is due to the different species from which they are derived, to the different transformation processes (radiation versus T-Ag) through which they were generated, or to other factors remains unknown. Interestingly, the MIN-6 cells displayed a particularly large percentage of cells in G2/M (Fig. 1A), consistent with either a particularly accelerated G1/S transition or a partial block at G2/M. Growth curves show that MIN6 cells grow at a rate comparable with BTC3 cells (Fig. 1B), suggesting that MIN-6 cells have acquired additional mutations delaying the G2/M transition, in addition to those involved in G1/S transition described herein. Importantly, the marked abnormalities in cell cycle control in these insulinoma cell lines mean that these cell lines must be used cautiously, if at all, as models of normal growth regulatory mechanisms.

We next comprehensively surveyed 32 regulatory proteins of the G1/S transition in each of the four insulinoma lines. This revealed important and unexpected findings. First, pRb was unexpectedly hypo- and not hyperphosphorylated in both of the rat insulinoma cell lines, those that have the most rapid proliferation rate. We hypothesized that this might result from pRb mutations that rendered pRb nonphosphorylatable. However, we easily were able to induce pRb phosphorylation in these same INS1 cells by combined overexpression of cdk-4 and cyclin D1. This indicates that pRb phosphorylation is not required for cell cycle progression in these cell lines. Second, despite their

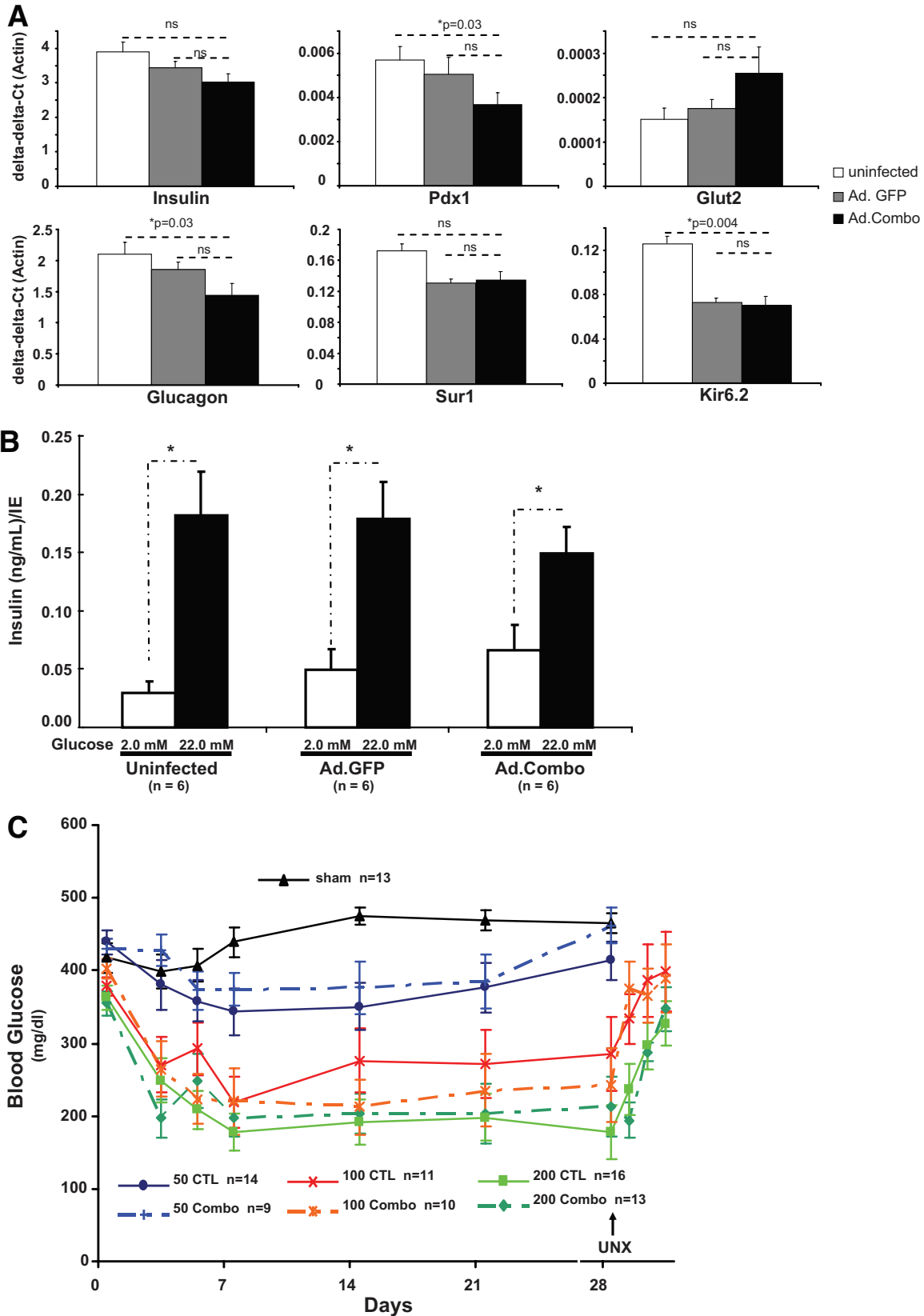


FIG. 8. The effect of combined overexpression of the seven cell cycle activators on rat islet cell differentiation markers, GSIS, and islet transplantation. **A:** Quantitative PCR was performed on the RNA of five to six different preparations of isolated rat islets transduced with virus as indicated. **B:** GSIS in uninfected and Ad.GFP- and Ad.combo-transduced islets exposed to 2.0 and 22 mmol/l glucose concentrations. *n* indicates the number of experiments performed, and each experiment was performed by triplicate. **P* < 0.05. **C:** Transplantation of rat islets into streptozotocin-induced diabetic NOD-SCID mice. Fifty, 100, or 200 IEs of normal nontransduced/islets or Ad.GFP-transduced islets served as controls (CTL) and were compared with Ad.Combo-transduced (Combo). Islets were transplanted under the mouse kidney capsule. Uninephrectomy (UNX) was performed 28 days after the transplant. "Sham" indicates the blood glucose in mice that had identical surgery but did not receive islets.

common origin, the two rat lines displayed important differences. The most striking of these is the abundance of cyclin D2 in INS1 cells, but the complete absence of cyclin D2 in RINm5F cells. Similarly, for the two mouse lines, MIN6 cells contain abundant cyclin D2, yet BTC3 cells completely lack cyclin D2. These observations are particularly noteworthy because cyclin D2 has been described as being essential for cell cycle progression for normal mouse β -cell replication (20,21). This clearly does not apply to rodent insulinomas. The existence of multiple molecular differences among cells that were derived from the similar or identical parental lines (MIN6 and BTC3; INS1 and RINm5F) is consonant with human cancers in which genomic instability with the consequent ongoing accumulation of multiple mutations is characteristic of transformed cells (8–10). It is also consistent with differing insertional mutagenic events in the separately derived RIP-Tag cell lines and/or different radiation mutagenic events in the two rat insulinoma cell lines. Third, cell cycle progression in the four rodent cell lines is accelerated despite a generalized increase in the upstream cell cycle inhibitors (the three pocket proteins, the four INK4s, and the three CIP/KIPs). We hypothesize that this reflects an eager, but futile, attempt to induce cell cycle arrest. Fourth, and most importantly, this survey reveals a marked increase in seven very plausible mediators of cell cycle progression: cdk1, cdk2, cdk4, cdk6, cyclin A, cyclin E, and cyclin D.

Importantly, despite the normal use of high glucose media for these cell lines, the upregulation of the seven proteins is not mediated by glucose levels: exposure to increased glucose concentrations induced mild upregulation of cyclin A, cyclin D3, and cyclin E in primary rat islets in agreement with previous reports (24) but not to the levels observed in insulinoma cell lines. Equally importantly, the changes observed in the rodent insulinoma cell lines were independent of cell cycle stage, occurring in randomly cycling cells, starvation-arrested cells, and synchronized cells. Collectively, these observations indicate that the upregulation of the seven pro-cell cycle molecules is an intrinsic feature of these cells, independent of cell cycle stage or nutritional status.

Proliferation of primary rat β -cells could be stimulated to levels observed in INS1 cells by forced expression of the seven cell cycle activators in combination. This was confirmed using FACS, Ki-67, and BrdU immunostaining.

Gratifyingly, the induced increase in proliferation had no effect on β -cell death. However, subtle changes in differentiation markers and GSIS *in vitro* were suggestive of β -cell dedifferentiation. To explore this issue more definitively, we introduced the Ad.combo into normal rat islets and observed their function *in vivo*. These studies can be interpreted in two ways. On one hand, Ad.combo did not adversely affect the apparent engraftment or net function of normal rat islets *in vivo*, suggesting that the Ad.combo was not intrinsically dedifferentiating in overall terms. Of course, because β -cell mass was not measured in the grafts, it is possible that β -cell mass was higher in the Ad.combo-transduced grafts and therefore that insulin secretion per β -cell was reduced. On the other hand, the proliferative advantage of the Ad.combo did not enhance the apparent overall function of the rat islets.

We interpret these *in vitro* and *in vivo* observations in several ways. First, although a combination of seven cell cycle molecules may be able to stimulate cell cycle progression *in vitro*, it is clear from Fig. 5B and C that the

addition of fewer pro-cell cycle molecules may suffice to stimulate proliferation effectively. We have recently reported that the addition of only two of these, cdk-6 and cyclin D1, clearly enhance the engraftment and function of human islets in the same *in vivo* model (31). Thus, evaluation of additional combinations of the seven cell cycle agonists *in vitro* and *in vivo* is warranted. This will be difficult, because the number of possible combinations is 7! (seven factorial), adjusted for random order, or ~ 120 . Second, adenovirus delivered at 250 MOI may have adversely affected β -cell function. Because cell cycle molecule-induced proliferation was maximized by 250 MOI (Fig. 5A and D), it is possible that lower MOI may permit the beneficial effects of cell cycle molecule delivery without the adverse effects. Third, although we have observed additivity among the seven members studied (Fig. 5B and C), we have not observed evidence of synergy among them: additional combinations may reveal such synergy. And fourth, aggressive replication rates may be unnecessary: slower replication rates may be perfectly satisfactory for expanding β -cell mass *in vitro* and *in vivo* while retaining differentiation/function. At a molecular level, the nature of a link between differentiation and proliferation, or even the existence of one in many cell types, is undefined. These observations suggest that there is an opportunity for further optimizing the MOI and duration of the transduction conditions and for further exploring the potential link between differentiation and proliferation in β -cells.

These studies increase available knowledge regarding cell cycle control in rodent insulinoma lines but also leave a number of questions unanswered. For example, they are confined to G1/S control molecules and leave unexplored other potential critically important pathways, such as telomere replication and extension, DNA stability, and signaling pathways upstream of the seven cell cycle molecules. Furthermore, although each of the seven candidates is reproducibly increased in the insulinoma lines and although overexpression of these seven in combination induces dramatic β -cell replication, these studies shed no light on which of these, if any, is actually responsible or essential for cell cycle progression in rodent insulinoma cell lines. Equally importantly, although each of the seven candidates is a normal cell cycle component, each has also been implicated in oncogenic contexts. An additional question relates to the use of adenovirus in particular, and gene therapy in general, as therapies for diabetes. Finally, and perhaps most importantly, whether the findings described herein in rodent islets will apply to human β -cell replication is unknown. These areas represent fertile ground for future study.

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REFERENCES

1. Cozar-Castellano I, Fiaschi-Taesch N, Bigatel TA, Takane KK, Garcia-Ocana A, Vasavada RC, Stewart AF: Molecular control of cell cycle

- progression in the pancreatic beta cell. *Endocrine Reviews* 27:356–370, 2006
2. Heit JJ, Karnik SK, Kim SK: Intrinsic regulators of pancreatic beta-cell proliferation. *Annu Rev Cell Dev Biol* 22:311–338, 2006
 3. Hohmeier H, Newgard CB: Cell lines derived from pancreatic islets. *Mol Cell Endocrinol* 228:121–128, 2004
 4. Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir GC, Lauris V: Continuous, clonal, insulin- and somatostatin-secreting cell lines established from transplantable rat islet cell tumor. *Proc Natl Acad Sci U S A* 77:3519–3523, 1980
 5. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167–178, 1992
 6. Efrat S, Linde S, Kofod H, Spector D, Delannoy M, Grant S, Hanahan D, Baekkeskov S: Beta-cell derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci U S A* 85:9037–9041, 1988
 7. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yakamura K: Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126–132, 1990
 8. Sherr CJ: Cancer cell cycles. *Science* 274:1672–1677, 1996
 9. Massague J: G1 cell-cycle control and cancer. *Nature* 432:298–306, 2004
 10. Weir BA, Woo MS, Getz G, Perner S, Ding L, Beroukhi R, Lin WM, Province MA, Kraja A, Johnson LA, Shah K, Sato M, Thomas RK, Barletta JA, Borecki IB, Broderick S, Chang AC, Chiang DY, Chirieac LR, Cho J, Fujii Y, Gazdar AF, Giordano T, Greulich H, Hanna M, Johnson BE, Kris MG, Lash A, Lin L, Lindeman N, Mardis ER, McPherson JD, Minna JD, Morgan MB, Nadel M, Orringer MB, Osborne JR, Ozenberger B, Ramos AH, Robinson J, Roth JA, Rusch V, Sasaki H, Shepherd F, Sougnez C, Spitz MR, Tsao MS, Twomey D, Verhaak RG, Weinstein GM, Wheeler DA, Winckler W, Yoshizawa A, Yu S, Zakowski MF, Zhang Q, Beer DG, Wistuba II, Watson MA, Garraway LA, Ladanyi M, Travis WD, Pao W, Rubin MA, Gabriel SB, Gibbs RA, Varmus HE, Wilson RK, Lander ES, Meyerson M: Characterizing the cancer genome in lung adenocarcinoma. *Nature* 450:893–898, 2007
 11. Garcia-Ocaña A, Takane KK, Reddy VT, Lopez-Talavera J-C, Vasavada RC, Stewart AF: Adenovirus-mediated hepatocyte growth factor transfer to murine islets improves pancreatic islet transplant performance and reduces beta cell death. *J Biol Chem* 278:343–351, 2003
 12. Becker TC, Noel RJ, Coats WS, Gomez-Foix AM, Alam T, Gerard RD, Newgard CB: Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol* 43:161–189, 1994
 13. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B: A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 95:2509–2514, 1998
 14. Cozar-Castellano I, Takane KK, Bottino R, Balamurugan AN, Stewart AF: Induction of β -cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenoviral delivery of cyclin-dependent kinase-4 and cyclin D₁. *Diabetes* 53:149–159, 2004
 15. Lopez-Talavera J-C, Garcia-Ocaña A, Sipula I, Takane KK, Cozar-Castellano I, Stewart AF: Hepatocyte growth factor gene therapy for pancreatic islets: reducing the minimal requisite islet transplant mass in a glucocorticoid-free rat model of allogeneic portal vein islet transplantation. *Endocrinology* 145:467–474, 2004
 16. Cozar-Castellano I, Haught M, Stewart AF: The cell cycle inhibitory protein, p21^{cip}, is not essential for maintaining β -cell cycle arrest or β -cell function in vivo. *Diabetes* 55:3271–3278, 2006
 17. Cozar-Castellano I, Weinstock M, Haught M, Velázquez-García S, Sipula D, Stewart AF: Comprehensive characterization of the G₁/S proteome in the islets of mice transgenic for hepatocyte growth factor, placental lactogen, or both: unique involvement of p21^{cip}. *Diabetes* 55:70–77, 2006
 18. Chung DC, Brown SB, Grahme-Cooke F, Seto M, Warshaw AL, Jensen RT, Arnold A: Overexpression of cyclin D1 occurs frequently in human pancreatic endocrine tumors. *J Clin Endocrinol Metab* 85:4373–4378, 2000
 19. Georgia S, Bhushan A: Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest* 114:963–968, 2004
 20. Kushner J, Ciemerych MA, Sicinska E, Wartschow LM, Teta M, Long SY, Sicinski P, White MF: Cyclins D2 and D1 are essential for postnatal pancreatic beta cell growth. *Mol Cell Biol* 25:3752–3762, 2005
 21. Martin J, Hunt SL, Dubus P, Sotillo R, Nehme-Pelluard F, Magnuson MA, Parlow AF, Malumbres M, Ortega S, Barbacid M: Genetic rescue of cdk-4-null mice restores pancreatic beta cell proliferation but not homeostatic cell number. *Oncogene* 22:5261–5269, 2003
 22. Ruzinova MB, Benezra R: Id proteins in development, cell cycle and cancer. *Trends Cell Biol* 13:410–418, 2003
 23. Kolly C, Suter MM, Muller EJ: Proliferation, cell cycle exit, and onset of terminal differentiation in cultured keratinocytes: pre-programmed pathways in control of c-myc and Notch1 prevail over extracellular calcium signals. *J Invest Dermatol* 124:1014–1025, 2005
 24. Teta M, Long SY, Wartschow LM, Rankin MM, Kushner JA: Very slow turnover of β -cells in aged adult mice. *Diabetes* 54:2557, 2005
 25. Alonso LC, Yokoe T, Zhang P, Scott DK, Kim SK, O'Donnell CP, Garcia-Ocaña A: Glucose infusion in mice: a new model to induce β -cell replication. *Diabetes* 56:1792–1801, 2007
 26. Bonner-Weir S, Deery D, Leahy JL, Weir GC: Compensatory growth of pancreatic β -cells in adult rats after short-term glucose infusion. *Diabetes* 38:49–53, 1989
 27. Bernard C, Thibault C, Berthault MF, Magnan C, Saulnier C, Portha B, Pralong WF, Penicaud L, Ktorza A: Pancreatic β -cell regeneration after 48-h glucose infusion in mildly diabetic rats is not correlated with functional improvement. *Diabetes* 47:1058–1065, 1998
 28. Paris M, Bernard-Kargar C, Berthault MF, Bouwens L, Ktorza A: Specific and combined effects of insulin and glucose on functional pancreatic beta-cell mass in vivo in adult rats. *Endocrinology* 144:2717–2727, 2003
 29. Steil GM, Trivedi N, Jonas JC, Hasenkamp WM, Sharma A, Bonner-Weir S, Weir GC: Adaptation of beta-cell mass to substrate oversupply: enhanced function with normal gene expression. *Am J Physiol Endocrinol Metab* 280:E788–E796, 2001
 30. Topp BG, McArthur MD, Finegood DT: Metabolic adaptations to chronic glucose infusion in rats. *Diabetologia* 47:1602–1610, 2004
 31. Fiaschi-Taesch M, Bigatel TA, Sicari B, Takane KK, Stewart AF: Overexpression of cdk-6, a unique human beta cell cdk, induces beta cell proliferation and promotes engraftment and function of human islets (Abstract). *Diabetes* 57 (Suppl. 1):A1, 2008